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The transcriptome of adult female *Anopheles darlingi* salivary glands

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Abstract

***Anopheles* (*Nyssorhynchus*) *darlingi* is an important malaria vector in South and Central America; however, little is known about molecular aspects of its biology. Genomic and proteomic analyses were performed on the salivary gland products of *Anopheles darlingi*. A total of 593 randomly selected, salivary gland-derived cDNAs were sequenced and assembled based on their similarities into 288 clusters. The putative translated proteins were classified into three categories: (S) secretory products, (H) housekeeping products and (U) products with unknown cell location and function. Ninety-three clusters encode putative secreted proteins and several of them, such as an anophelin, a thrombin inhibitor, apyrases and several new members of the D7 protein family, were identified as molecules involved in haematophagy. Sugar-feeding related enzymes (α-glucosidases and α-amylase) also were found among the secreted salivary products. Ninety-nine clusters encode housekeeping proteins associated with energy metabolism, protein synthesis, signal transduction and other cellular functions. Ninety-seven clusters encode proteins with no similarity with known proteins. Comparison of the sequence divergence of the S and H categories of proteins of *An. darlingi* and *An. gambiae* revealed that the salivary proteins are less conserved than the housekeeping proteins, and therefore are changing at a faster evolutionary rate. Tabular and supplementary material containing the cDNA sequences and annotations are available at http://www.ncbi.nlm.nih.gov/projects/Mosquito/A_darlingi_sialome/**

Keywords: *Anopheles darlingi*, salivary glands, proteome, transcriptome.

Introduction

The mosquito *Anopheles darlingi* (subgenus *Nyssorhynchus*) is an important vector of human malaria in South and Central America (Deane, 1986; Rubio-Palis & Zimmerman, 1997). The incidence of malaria has grown in this area during the last 30 years, attaining over a million cases annually, and in Brazil alone, four to five hundred thousand cases have been reported every year over the last decade (PAHO, 1998). The absence of an effective malaria vaccine, and the spread of drug-resistant *Plasmodium* parasites as well as insecticide resistance in vector populations (Crampton *et al.*, 1992), means there is urgent need for novel malaria control strategies. Rational approaches to new strategies are anticipated to originate from studies of insect physiology, immunology, biochemistry and molecular biology. These approaches will benefit from detailed understandings of interactions that occur between parasites and insects in all of these disciplines (Hurd, 1994).

Despite its importance as a malaria vector, little is known regarding the genome and proteome of *Anopheles darlingi*, mainly as a result of the inability to develop laboratory-adapted strains of the mosquito. Here we describe the transcriptome from the salivary glands of wild-caught female *An. darlingi*. We have selected the salivary glands as the organ to be studied because of its direct involvement in the transmission of malaria parasites to human hosts (Kappe *et al.*, 2003). Generation of a set of *An. darlingi* salivary gland cDNAs and deduced proteins provides indispensable tools for the systematic and comprehensive analysis of molecules that may play an active role in mosquito blood feeding and the pathogenesis of malaria.

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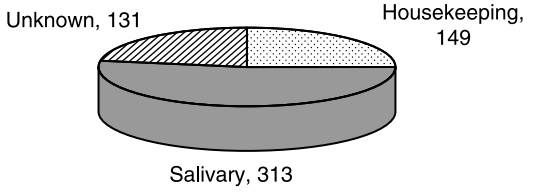
Results and discussion

Organization of transcriptome information

A total of 593 cDNA inserts were sequenced from a *An. darlingi* salivary gland cDNA library and these were assembled by the CAP program (Huang, 1992) into 289 clusters of related sequences. Using the BLAST package of programs (Altschul *et al*., 1997), we compared sequences for each cluster in the database with the nonredundant protein and nucleotide sets of the NCBI and Gene Ontology databases (Ashburner *et al*., 2000; Lewis *et al*., 2000; Hvidsten *et al*., 2001). Translated sequences also were screened with RPSBlast for protein motifs of the combined set of Pfam (Bateman *et al*., 2000) and SMART (Schultz *et al*., 2000) databases (also known as the Conserved Domains Database [CDD]). The sequences also were compared with the proteome set of the mosquito *An. gambiae* (available for FTP download at NCBI and other sites).

Finally, we submitted all translated sequences (starting with the first Met) to the Signal P server (Nielsen *et al*., 1997) to detect N-terminal amino acid sequences indicative of secretion signal peptides. With this information, the clustered database was annotated and classified into three categories of clusters: S, those associated with possible secreted products; H, those possibly associated with 'housekeeping' functions (metabolic activities anticipated in all cell types); and U, those of unknown function. Accordingly, ninety-nine cDNA clusters containing a total of 149 sequences (25.12% of the transcriptome) were classified as category H (Fig. 1). These clusters have an average of 1.5 sequences per cluster. This contrasts with the ninety-three clusters containing 313 sequences (52.78% of the transcriptome; average of 3.6 sequences per cluster) classified as category S. These average cluster sizes are statistically different from one another ($P < 0.01$, χ^2 test), and consistent with what was observed in the salivary gland transcriptomes of *Ae. aegypti*, *An. gambiae*, *An. stephensi* and *Ixodes scapularis* (Francischetti *et al*., 2002b; Valenzuela *et al*., 2002b,c, 2003). Finally, ninety-seven clusters with 131 sequences (22.09% of the transcriptome; average of 1.3 sequences per cluster) were classified as category U.

A



B

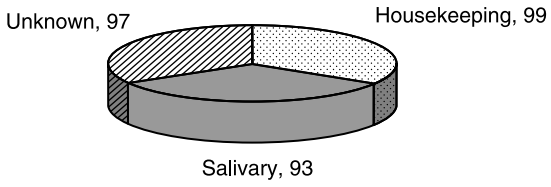


Figure 1. Numbers of sequences (A) or clusters (B) obtained from the 593 randomly selected clones from the adult female *Anopheles darlingi* salivary glands cDNA library. The sequences were classified as housekeeping, secretory or unknown functions. Absolute numbers of sequences or clusters are indicated on the diagrams.

Preliminary characterization of the salivary gland proteome of An. darlingi

Parallel to the analysis of the salivary gland transcriptome of *An. darlingi*, sequence information was obtained for some of the most abundant proteins in the salivary glands of adult female mosquitoes. Proteins from fifteen salivary gland pairs were separated by SDS-PAGE, transferred to PVDF membranes, stained and the major polypeptides submitted to Edman degradation. Six polypeptides yielded useful information, five of which could be assigned to protein sequences predicted from our cluster database (Fig. 2). Other molecules did not yield useful amino acid sequences, either because they were blocked at their aminoterminal ends or because the Edman degradation resulted in uninformative low signals. The five clusters associated with the amino acid sequences generated from the Edman degradation reactions had between three and eleven cDNA sequences, with an average of six sequences per cluster, twice the average of the clusters in the S group. This result is consistent with the interpretation that the quantity of a particular protein correlates with its mRNA abundance. A good correlation between protein abundance and mRNA abundance was also observed in other analysed organisms (Futcher *et al*., 1999).

Description of secretory (S) category clusters

Ninety-three clusters of sequences belonging to the S category were identified (Table 1). These clusters contain 1–44 cDNA sequences each and most belong to well-known families of proteins, although some do not have a known function.

D7-related proteins

D7-related proteins, named after the prototype *Ae. aegypti* D7 protein (James *et al*., 1991), comprise a unique family found in the salivary glands of mosquitoes and sand flies, and are related distantly to the insect odorant-binding proteins (Hekmat-Scafe *et al*., 2000; Calvo *et al*., 2002; Valenzuela *et al*., 2002a). Two classes of D7-related proteins have been described: long (28–30 kDa), found in both mosquitoes and sand flies; and short (15–20 kDa), found so far only in mosquitoes (Arca *et al*., 1999; Calvo *et al*., 2002; Valenzuela *et al*., 2002a; Malafronte *et al*., 2003). Nine

Table 1. *An. darlingi* salivary glands cDNA clusters encoding for proteins probably secreted

No. of sequences	Assembled contig	*E* value (best match to NR protein database)	Best match to AGPROT database	Percentage identity	Comments (similar to/putative function)
Allergen/Antigen-5 related protein					
14	AD-contig_214	1e–66	CRA|agCP8743	58	30 kDa allergen
9	AD-contig_66	3e–82	CRA|agCP6145	75	antigen-5
D7 proteins					
44	AD-contig_1	2e–67	CRA|agCP11198	61	D7-related
11	AD-contig_230	5e–20	CRA|agCP11220	36	D7-related
9	AD-contig_279	3e–23	CRA|agCP11196	36	D7-related
7	AD-contig_77	5e–13	CRA|agCP11196	28	D7-related
5	AD-contig_159	2e–82	CRA|agCP10845	50	D7-related
4	AD-contig_229	4e–20	CRA|agCP11220	37	D7-related
2	AD-contig_256	3e–7	CRA|agCP11220	28	D7-related, short
1	AD-contig_228	6e–12	CRA|agCP2228	42	D7clu5 short form (hamadarin)
1	AD-contig_278	6e–21	CRA|agCP11220	40	short form D7clu5
Enzymes and enzymes inhibitors linked to blood and sugar meals					
5	AD-contig_137	4e–33	CRA|agCP11208	43	anophelin
2	AD-contig_253	4e–78	CRA|agCP9757	49	apyrase
3	AD-contig_217	4e–75	CRA|agCP10591	81	apyrase (full clone)
3	AD-contig_224	1e–82	CRA|agCP10591	54	apyrase (truncated clone)
1	AD-contig_45	2e–32	CRA|agCP7190	50	salivary peroxidase
1	AD-contig_115	1e–11	CRA|agCP14623	38	thrombin inhibitor infestin precursor
6	AD-contig_88	1e–115	CRA|agCP12790	58	maltase
1	AD-contig_265	1e–82	CRA|agCP12790	82	maltase-like protein
1	AD-contig_65	4e–21	EBI|8952	54	maltase-like protein
1	AD-contig_106	1e–24	CRA|agCP1208	36	alpha-amylase
1	AD-contig_89	1e–6	CRA|agCP12790	37	probable maltase precursor
1	AD-contig_124	2e–17	CRA|agCP12065	49	salivary glucosidase
15	AD-contig_123	9e–32	CRA|agCP12065	49	salivary glucosidase
4	AD-contig_125	2e–17	CRA|agCP12065	37	salivary glucosidase
Mucin-like proteins					
8	AD-contig_242	1e–17	CRA|agCP1772	51	mucin
5	AD-contig_244	5e–16	CRA|agCP1772	40	mucin
3	AD-contig_220	1e–10	CRA|agCP7687	31	mucin
1	AD-contig_243	1e–17	CRA|agCP1772	38	mucin-like
1	AD-contig_51	2e–59	CRA|agCP3409	67	peritrophin 1 (mucin-like peritrophin)
1	AD-contig_34	4e–70	CRA|agCP14528	70	mucin?
Possibly related to immunity					
10	AD-contig_266	4e–15	CRA|agCP7505	51	cecropin
5	AD-contig_181	9e–24	CRA|agCP7503	50	cecropin
4	AD-contig_203	3e–41	CRA|agCP6915	78	defensin
2	AD-contig_239	1e–22	EBI|7267	32	putative infection responsive short peptide
1	AD-contig_129	3e–17	CRA|agCP7503	67	antibiotic peptide cecropin A2
1	AD-contig_166	0.012	CRA|agCP14093	85	putative gram negative bacteria binding protein
1	AD-contig_267	2e–15	CRA|agCP7505	45	cecropin CecC
1	AD-contig_180	2e–81	CRA|agCP9741	64	T-cell immunomodulatory protein
1	AD-contig_59	2e–38	CRA|agCP3859	57	lysozyme
SG family of anopheline salivary proteins					
3	AD-contig_199	2e–18	CRA|agCP13537	37	gSG1b
7	AD-contig_28	0.084	CRA|agCP6138	32	gSG2
1	AD-contig_29	0.044	CRA|agCP6138	31	gSG2 protein
3	AD-contig_207	1e–29	CRA|agCP11109	44	gSG7
1	AD-contig_206	1e–22	CRA|agCP11109	41	gSG7
2	AD-contig_247	1e–24	CRA|agCP2222	54	gSG7
4	AD-contig_202	3e–31	CRA|agCP7185	54	gSG8
11	AD-contig_255	1e–20	CRA|agCP13467	32	SG1 family
16	AD-contig_201	2e–29	CRA|agCP6430	42	SG3 family
Chitinase					
1	AD-contig_40	6e–26	CRA|agCP6090	55	chitinase
Similar to previously described salivary An. gambiae proteins of unknown function					
5	AD-contig_16	1e–6	CRA|agCP13582	38	hypothetical protein 15
5	AD-contig_192	0.012	CRA|agCP15011	52	no match in Ag (nonannotated protein)
1	AD-contig_35	6e–28	CRA|agCP8839	33	putative 53.7 kDa salivary protein
2	AD-contig_15	1e–4	CRA|agCP13582	45	unknown
1	AD-contig_24	7e–8	EBI|4655	29	pectinesterase
1	AD-contig_78	5e–4	CRA|agCP11977	35	unknown
5	AD-contig_100	3e–24	CRA|agCP8099	41	unknown
1	AD-contig_189	7e–17	CRA|agCP11339	93	unknown

Table 1. (*Continued*)

No. of sequences	Assembled contig	*E* value (best match to NR protein database)	Best match to AGPROT database	Percentage identity	Comments (similar to/putative function)
1	AD-contig_285	1e–4	CRA|agCP13137	56	unknown
1	AD-contig_281	3e–33	CRA|agCP3858	51	unknown
1	AD-contig_276	5e–28	CRA|agCP1678	53	unknown
1	AD-contig_87	7e–7	CRA|agCP2550	53	unknown
1	AD-contig_262	1e–37	CRA|agCP2969	73	unknown
1	AD-contig_73	9e–10	CRA|agCP11977	45	unknown
1	AD-contig_274	6e–27	CRA|agCP15376	52	unknown
1	AD-contig_288	7e–62	CRA|agCP12405	88	unknown
1	AD-contig_169	8e–4	CRA|agCP1607	78	unknown
1	AD-contig_67	2e–4	CRA|agCP4709	38	unknown
1	AD-contig_39	5e–8	CRA|agCP7243	90	unknown
1	AD-contig_136	5e–13	CRA|agCP5458	54	unknown
1	AD-contig_198	0.092	EBI|6164	46	unknown
1	AD-contig_30		CRA|agCP6138	31	unknown
1	AD-contig_10		CRA|agCP11711	30	unknown
Putative secreted proteins with unknown function					
6	AD-contig_3	0.003	No match		hypothetical salivary protein 8.2
4	AD-contig_2	0.003	No match		hypothetical salivary protein 8.2
1	AD-contig_68	0.002	No match		unknown
1	AD-contig_71	0.035	No match		unknown
1	AD-contig_69	2e–4	No match		unknown
1	AD-contig_58		No match		unknown
1	AD-contig_286		No match		unknown
1	AD-contig_111		No match		unknown
1	AD-contig_158		No match		unknown
4	AD-contig_209		No match		unknown
1	AD-contig_114		No match		unknown
1	AD-contig_116		No match		unknown
1	AD-contig_134		No match		unknown
1	AD-contig_144		No match		unknown
1	AD-contig_154		No match		unknown
1	AD-contig_284		No match		unknown
1	AD-contig_72		No match		unknown
1	AD-contig_135		No match		unknown
1	AD-contig_63		No match		unknown
1	AD-contig_44		No match		unknown

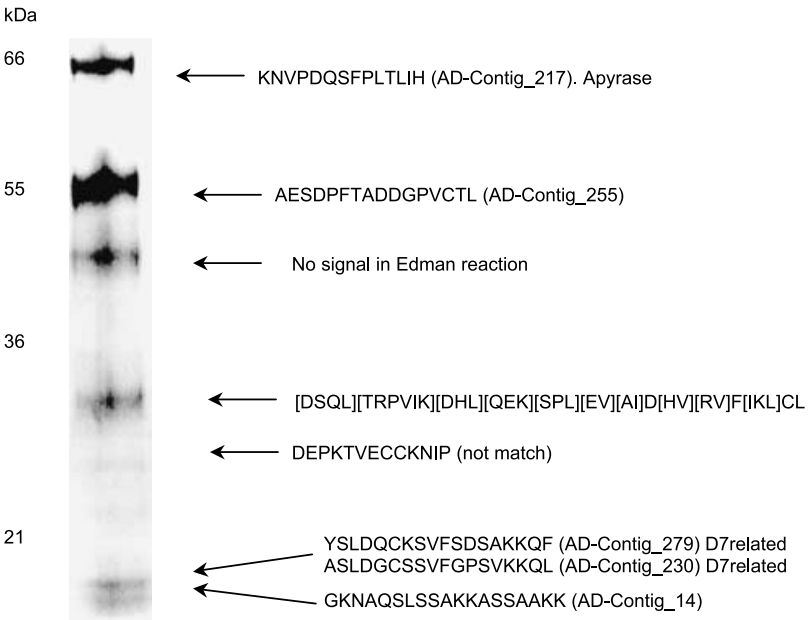


Figure 2. SDS-PAGE of *Anopheles darlingi* salivary gland proteins. Molecular weight markers are shown to the left and the amino acid sequences obtained by Edman degradation to the right. The corresponding cluster is indicated next to each amino acid sequence.

clusters coding for proteins of the D7 family were identified in the *An. darlingi* salivary gland transcriptome, indicating that several D7-related genes exist in the genome of this mosquito. The occurrence of multiple D7-related genes and their organization in the genome in *An. gambiae* was reported previously (Arca *et al*., 2002). The most abundant cluster (forty-four sequences) codes for the recently described protein, Anda-D7r3-2 (Calvo *et al*., 2002). Anda-D7r3-2 has 61% amino acid identity to the reported short-form D7clu2 salivary protein of *An. gambiae* (CRA|agCP11198) and 14% identity to the long D7 protein of *Ae. aegypti* (gi|159559) (James *et al*., 1991).

The D7-related proteins may inhibit activation of host plasma, as was observed for hamadarin, a D7-related protein of *An. stephensi* saliva recently characterized as an inhibitor of Factor XII (Isawa *et al*., 2002). In addition, they may have different functions, as is the case with *Rhodnius* salivary lipocalins (Montfort *et al*., 2000). Several closely related proteins of the lipocalin family exist in the saliva of *R. prolixus*, each of which has a different antihaemostatic activity (Andersen *et al*., 2002; Francischetti *et al*., 2002a). This diversity probably reflects a scenario of gene duplication and divergence of function (Sankoff, 2001).

Consistent with the abundance of cDNA sequences in the D7 family of clusters, two of eight major *An. darlingi* salivary gland proteins produced amino-terminal sequences matching D7-related proteins (Fig. 2). The sequence, ASLDGCSS, found in the 16–18 kDa region of the gel, matches the previously reported D7clu2 of *An. gambiae* (Francischetti *et al*., 2002b). The other amino-terminal sequence, YSLDQCKS, matches proteins of the previously reported *An. gambiae* D7clu5 protein (Francischetti *et al*., 2002b). No function has been discovered yet for these proteins. The abundance of D7 proteins observed by SDS-PAGE also is consistent with prior findings in which D7 was isolated as a major protein in the acidic soluble fractions of *An. darlingi* salivary glands (Calvo *et al*., 2002).

Mucins

The most abundant cluster in this group (eight clones sequenced) codes for a protein with 50% identity to a putative salivary mucin of *An. stephensi* (AAO06835). This protein also is similar to the Trypanosomal mucin-like glycoproteins (Di Noia *et al*., 1998). Molecules belonging to this family of trypanosomal proteins resemble vertebrate mucins and their amino acid sequences consist of three regions. The amino and carboxyl termini are conserved among all members of the family, whereas the central region is not well conserved and contains a large number of threonine residues, some of which can be glycosylated. Indirect evidence is interpreted to suggest that these genes might encode the core protein of parasite mucins, glycoproteins that may be involved in the interaction with, and invasion of, mammalian host cells (Hansen *et al*., 1998).

Five other clusters also were identified tentatively as encoding mucin-like proteins (Table 1). These components of mosquito saliva could function as lubricants of the salivary canal and also could have other activities such as modulation of macrophages, as is the case with surface mucins of *Trypanosoma cruzi* (Acosta-Serrano *et al*., 2001; Ropert *et al*., 2002).

Antigen 5-related proteins

Proteins related to this family are found in the venom glands of Hymenoptera (Henriksen *et al*., 2001) and in the salivary glands of sand flies (Charlab *et al*., 1999), tsetse flies (Li *et al*., 2001) and mosquitoes (Francischetti *et al*., 2002b; Valenzuela *et al*., 2002c). The *An. gambiae* antigen-5 protein is expressed specifically in the medial lobes of the female salivary glands of that mosquito (Arca *et al*., 1999). Antigen-5-related salivary products are members of a group of secreted proteins that belong to the CAP family (cysteine-rich secretory proteins; antigen-5 proteins of insects; pathogenesis-related protein 1 of plants) (Megraw *et al*., 1998). The CAP family is related to venom allergens in social wasps and ants (Hoffman, 1993) and to antifungal proteins in plants (Stintzi *et al*., 1993; Szyperski *et al*., 1998). One cDNA cluster, with nine clones sequenced, encodes a protein with similarity to other antigen-5-related molecules (Table 1). The *An. darlingi* antigen-5-related protein has > 75% identity to *An. gambiae* agCP6145 and is 74% identical to the salivary antigen-5-related protein 1 of the same mosquito (Francischetti *et al*., 2002b).

The general function for CAP proteins is controversial (Schreiber *et al*., 1997). Some of the CAP family proteins function as protease inhibitors (Megraw *et al*., 1998). As for the physiology of blood-sucking insects, these molecules could represent an adaptive response to inhibit coagulation, complement activation or any other component of the vertebrate host haemostasis potentially harmful to the mosquitoes. However, proteins from this family have also been associated with proteolytic activity in *Echinococcus granulosus* (Lorenzo *et al*., 2003) and *Conus textile* (Milne *et al*., 2003).

30 kDa antigen

One of the most abundant clusters (fourteen sequences) encodes a product similar to the protein agCP8743 of *An. gambiae* (58% identity) and the salivary 30 kDa protein gi|18389879 (47% identity) of the same mosquito. This mosquito salivary protein was first described in *Ae. aegypti* mosquitoes (Simons & Peng, 2001) and later found to be in the salivary glands of *An. gambiae* (Francischetti *et al*., 2002b). It has a long region of low amino acid complexity, consisting mainly of Gly and Glu residues. The clone identified here may represent the homologous protein in *An. darlingi*. The function of this protein is unknown.

The SG family

This family of anopheline salivary gland proteins, described as SG or gSG proteins (Arca *et al*., 1999; Lanfrancotti *et al*., 2002), does not yield significant similarities (by BLASTP) to other proteins in the NCBI database, except among its own members. This family also includes the distantly related salivary *An. gambiae* TRIO protein (Francischetti *et al*., 2002b). TRIO is a multidomain protein that binds the lymphocyte-activating receptor transmembrane tyrosine phosphatase (PTPase) and contains a protein kinase domain. It was proposed that TRIO may orchestrate cell–matrix and cytoskeletal rearrangements necessary for cell migration (Lin & Greenberg, 2000).

Two other cDNA clusters (fourteen sequences) of the *An. darlingi* salivary gland library yielded similarities to *An. gambiae* proteins annotated as members of the SG1 family (Table 1). All proteins belonging to this family, including the conceptual product of the SG1-like polypeptide identified from *An. darlingi* cDNA clones, have a clear signal peptide indicative of secretion.

The *An. darlingi* transcriptome contains two clusters of cDNA (eight sequences) having sequence similarity to *An. gambiae* SG2 and gSG2 proteins (Table 1). This family, first described in *An. gambiae* as SG2, gSG2 and SG2-like protein (Arca *et al*., 1999; Lanfrancotti *et al*., 2002), consists of proteins 114–168 amino acids in length that are rich in Gly or Asn. High protein similarity matches in the NCBI database are only produced among those anopheline proteins that belong to the SG2 group. These two clusters may represent an mRNA corresponding to the *An. darlingi* gene homologous to the *An. gambiae* SG2 gene. It is interesting that *Ixodes scapularis* salivary glands also contain glycine-rich peptides of equivalent size (Valenzuela *et al*., 2002b). Their function also is unknown.

Finally, we found three clusters (twenty-four sequences) having similarity to other proteins of the SG salivary protein families (gSG3, gSG7 and gSG8) of *An. gambiae*. This subgroup of proteins is the second most abundant in the *An. darlingi* transcriptome. Proteins of the SG family, by being unique to anopheline mosquitoes, may become a useful immunological marker for exposure to this mosquito group by humans and animals.

Enzymes and inhibitors associated with antihaemostatic activities

Six clusters (twenty-five sequences), with an average of 2.5 clones sequenced per cluster, encode products that are similar to enzymes and enzyme inhibitors related to haematophagy. Salivary peroxidases, which act as vasodilators, were found in the salivary glands of anopheline mosquitoes (Ribeiro & Nussenzveig, 1993; Ribeiro *et al*., 1994; Ribeiro & Valenzuela, 1999). One cDNA cluster, consisting of only one sequence, matches the *An. albimanus* salivary peroxidase and may be related to the *An. gambiae* protein agCP7190.

Three different clusters (eight sequences) encode proteins having 49–89% identity to the *An. gambiae* salivary apyrase (agCP10591). One cluster, containing three sequences, represents truncated clones matching the apyrase gene product. In support of the existence of salivary apyrases in *An. darlingi*, an aminoterminal sequence (KNVPDQ) that matches the putative salivary apyrase gene product was found in the 60 kDa region of the SDS-PAGE of salivary glands (Fig. 2). Furthermore, apyrase activity was described previously in the salivary glands of this mosquito (Moreira-Ferro *et al*., 1999). Apyrases are a polyphyletic group of enzymes found ubiquitously in the salivary glands of blood-feeding insects and ticks. Apyrases degrade the neutrophil-inducing substance ATP, and the platelet-aggregating nucleotide ADP to AMP, presumably facilitating blood feeding. In *Ae. aegypti*, apyrase is a member of the 5′-nucleotidase family (Champagne *et al*., 1995). In *An. gambiae*, two such genes are expressed in the salivary glands and annotated as apyrase and 5′-nucleotidase; however, both could actually be coding for proteins with apyrase activity (Arca *et al*., 1999; Lombardo *et al*., 2000).

The *An. darlingi* salivary transcriptome has one cDNA cluster with five clones sequenced encoding a protein similar to antithrombins of the anophelin family (Valenzuela *et al*., 1999; Francischetti *et al*., 1999). The putative *An. darlingi* anophelin peptide is 43%, 41% and 86% identical to the homologues of *An. gambiae*, *An. stephensi* and *An. albimanus*, respectively.

Another cluster within the *An. darlingi* transcriptome encodes a protein with 38% identity to the *An. gambiae* agCP14623 protein and 52% identity to the thrombin inhibitor, infestin, of *Triatoma infestans* (Campos *et al*., 2002). The infestin gene encodes a protein with four nonclassical Kazal-type domains with an apparent molecular mass of 22 kDa, and belongs to a family of serine protease inhibitors. This protein, found in the *T. infestans* midgut, showed inhibitory activities towards thrombin and trypsin (Campos *et al*., 2002). Surprisingly, infestin inhibited not only thrombin and trypsin, but also factor XIIa, factor Xa and plasmin, inhibiting blood clotting during the blood meal.

Sugar-meal digestion

Mosquito salivary glands secrete enzymes such as maltases or α-glucosidases (*Maltase-like 1* [*Mal*l] gene, James *et al*., 1989; Marinotti *et al*., 1990) and possibly α-amylases (*Amy*l gene, Grossman & James, 1993) that help in sugar-meal digestion. The *An. darlingi* transcriptome has eight clusters, represented by thirty sequences, with similarity to enzymes linked to sugar digestion in other mosquitoes.

Four different clusters (nine sequences) showed similarity to the *Mal*l gene of *Ae. aegypti* (James *et al*., 1989) and to the *An. gambiae* protein agCP12790. This is consistent

with the finding that the α-glucosidase activity has been demonstrated in the salivary glands of *An. darlingi* (Moreira-Ferro *et al*., 1999). *Anopheles stephensi* also expresses four gene products related to maltases in its salivary glands (Valenzuela *et al*., 2003).

Additionally, we sequenced one clone with similarity to α-amylases of *Ae. aegypti* (Grossman & James, 1993) and 36% identity with the protein agCP1208 of *An. gambiae*. In *Ae. aegypti*, the *Amylase* I gene (*Amy*I) is expressed specifically in the salivary glands and its function has been proposed to be involved with carbohydrate metabolism. However, amylase activity is detected at a very low level in *Ae. aegypti* salivary gland extracts (Grossman & James, 1993).

Finally, in this group we sequenced twenty clones included in three different clusters coding for proteins with similarity to salivary glucosidases. The most abundant cluster in this group has fifteen sequences with 49% identity to the *An. gambiae* protein agCP12065 and 52% identical to the cellulose 1,4-beta-cellobiosidase from *Xylella fastidiosa* (NP_778753). This protein also could be involved in sugar digestion in *An. darlingi* mosquitoes.

Putative immunity-related products

Four clusters, ranging from one to ten sequences in each cluster, encode proteins with similarity to the antimicrobial peptide Cecropin family (Cec-A, -B and -C) of *An. gambiae* (Vizioli *et al*., 2000; Zheng & Zheng, 2002). Cecropins are small antibacterial peptides first isolated from the lepidopteran *Hyalophora cecropia* (Steiner *et al*., 1981), and have since been isolated from various insects, such as *Drosophila melanogaster* (Kylsten *et al*., 1990), *Ae. aegypti* (Lowenberger *et al*., 1999) and *An. gambiae* (Vizioli *et al*., 2000). Cecropins and their derivatives have a wide range of antimicrobial targets, including *Candida albicans* (Park *et al*., 1997) to *Plasmodium* parasites (Gwadz *et al*., 1989; Rodriguez *et al*., 1995), *Trypanosoma cruzi* (Barr *et al*., 1995), *Leishmania* (Akuffo *et al*., 1998) and the filarial worm, *Brugia pahangi* (Chalk *et al*., 1995). Cecropin expression is induced by bacterial challenge, but may be produced constitutively in the salivary glands to protect the sugar meal from microbial fermentation.

Another cluster, with four sequences, encodes a peptide similar to *An. gambiae* Defensin (78% identity). The expression of Defensin is predominantly induced in the mosquito fat body shortly after bacterial challenge. It also is induced locally in the midgut and salivary gland epithelia upon invasion by malaria parasites, suggesting that Defensin may have a broad role in the defence against both microbes and parasites (Richman *et al*., 1996, 1997).

Lysozyme, an antibacterial enzyme first described as a mosquito salivary activity in *Ae. aegypti* (Rossignol & Lueders, 1986), also was found in salivary glands of *An. darlingi* (Moreira-Ferro *et al*., 1998). Only one cluster with one sequence is similar to the *An. gambiae* LYC_ANOGA lysozyme precursor (66% identity). This result is in contrast with the *An. stephensi* transcriptome (Valenzuela *et al*., 2003), where an abundant cluster (fourteen sequences) was found. Salivary lysozyme may help to deter bacterial growth in sugar meals of mosquitoes, which are stored in the crop.

Three additional clusters observed in the mosquito salivary glands that encode putative secreted proteins may be involved with immunity. One cluster (one sequence) encodes a product similar to the *An. gambiae* protein (AgCP14093) and also similar to Gram-negative binding protein (AAM73871), the second cluster (two sequences) encodes a putative infection-responsive short peptide, similar to the gambicins of *An. gambiae* (Vizioli *et al*., 2001) and *Culex pipiens* (AA038515), and the third cluster (one sequence) encodes a product similar to the *An. gambiae* agCP9741 protein (64% identity) and also is similar to a T-cell immunomodulatory protein of *Homo sapiens*.

Expression of the mRNAs encoding Gram-negative bacteria binding proteins in mosquito salivary glands was described for *An. gambiae* (Dimopoulos *et al*., 1997) and *Ae. aegypti* (Valenzuela *et al*., 2002c). Gambicin was first described in *An. gambiae* by Vizioli *et al*. (2001). This molecule is an antimicrobial peptide that lacks sequence homology with other known immune-related proteins. The mature peptide can kill both Gram-positive and Gram-negative bacteria. The T-cell immunomodulatory protein function in *An. darlingi* salivary glands is unknown; however, immunomodulatory activity in the saliva of the mosquito *Ae. aegypti* has been described (Cross *et al*., 1994).

Description of secretory products with unidentified function in An. darlingi

The *An. darlingi* salivary gland cDNA library yielded twenty-three clusters of sequences similar to predicted proteins from the *An. gambiae* genome but not previously described in salivary gland transcriptomes. These hypothetical proteins do not yield significant matches to other proteins in the NR protein database and are yet to be characterized.

Furthermore, twenty cDNA clusters, with thirty-one clones sequenced, encode proteins predicted to have a signal peptide indicative of secretion; however, these do not match other known proteins, even when the BLAST filter to exclude low-complexity sequences was removed. When the protein sequences were compared with the *An. gambiae* genome (using tblastn), they produced no matches, indicating that these are novel proteins or truncated clones.

Description of housekeeping (H) category clusters

Of the ninety-nine H category clusters in the salivary transcriptome of *An. darlingi* (Table 2), forty-five clusters correspond to genes involved in protein synthesis and secretion including those that encode rRNA, ribosomal proteins, mitochondrial proteins and Golgi vesicular membrane trafficking proteins. Twenty-two clusters (thirty-six clones

Table 2. *An. darlingi* salivary glands cDNA clusters encoding proteins associated with housekeeping function

No. of sequences	Assembled contig	*E* value (best match to NR protein database)	Best match to AGPROT database	Percentage identity	Comments identity (similar to/putative function)
Heat shock-like protein					
1	AD-contig_32	1e–63	CRA|agCP3435	79	heat shock protein
2	AD-contig_246	3e–49	CRA|agCP12309	95	heat shock protein cognate 4
1	AD-contig_191	3e–21	CRA|agCP12309	67	heat shock-like protein
1	AD-contig_81	6e–42	CRA|agCP11787	92	chaperonin containing TCP1
1	AD-contig_128	2e–27	CRA|agCP11981	91	chaperonin-heat shock
Metabolism related proteins					
1	AD-contig_121	1e–15	CRA|agCP8069	74	*N*-acetyltransferase
1	AD-contig_57	6e–47	CRA|agCP12915	55	ADP-ribosylation-like factor 6
1	AD-contig_104	1e–82	CRA|agCP8658	70	dimeric dihydrodiol dehydrogenase
1	AD-contig_31	5e–39	CRA|agCP8498	95	glyceraldehyde-3-phosphate dehydrogenase
1	AD-contig_287	4e–41	CRA|agCP10011	75	glycosylasparaginase
1	AD-contig_119	5e–59	CRA|agCP3334	92	glutamate carboxypeptidase-like
1	AD-contig_289	1e–63	CRA|agCP15559	96	NADH dehydrogenase (ubiquinone)
1	AD-contig_76	2e–36	CRA|agCP3297	97	NADH dehydrogenase (ubiquinone)
1	AD-contig_85	9e–65	CRA|agCP3151	82	NADH dehydrogenase (ubiquinone)
1	AD-contig_82	6e–29	CRA|agCP2296	86	NADH dehydrogenase (ubiquinone)
1	AD-contig_46	2e–70	CRA|agCP6292	84	*N*-methyl-D-aspartate receptor-associated protein
1	AD-contig_11	2e–53	CRA|agCP1214	91	peptidylprolyl cis-trans isomerase
1	AD-contig_268	3e–99	CRA|agCP3166	94	peroxidase (antioxidant enzyme)
1	AD-contig_48	5e–42	EBI|9772	87	peroxidase (antioxidant enzyme)
1	AD-contig_60	9e–73	EBI|2906	72	phosphotidylinositol 3 kinase
1	AD-contig_26	2e–25	CRA|agCP6680	49	polyhydroxyalkanoate synthesis protein
1	AD-contig_13	9e–24	CRA|agCP10881	52	similar to programmed cell death
1	AD-contig_261	8e–92	CRA|agCP3730	89	succinate dehydrogenase B
1	AD-contig_151	2e–64	CRA|agCP4843	72	ubiquinol-cytochrome C reductase
4	AD-contig_200	1e–44	CRA|agCP13749	59	ubiquitin
1	AD-contig_168	2e–28			NADH dehydrogenase subunit
1	AD-contig_161	0.011			NADH2 dehydrogenase (ubiquinone)
2	AD-contig_238	4e–31			ATP-synthase
1	AD-contig_36	3e–79			cytochrome b oxydase
5	AD-contig_148	4e–65			cytochrome oxydase
3	AD-contig_221	5e–41			cytochrome b oxydase
Protein synthesis and secretion					
4	AD-contig_208	1e–40	CRA|agCP1641	81	ribosomal protein
2	AD-contig_235	1e–71	CRA|agCP14911	87	ribosomal protein
1	AD-contig_146	4e–58	CRA|agCP11398	56	ribosomal protein
2	AD-contig_231	5e–42	CRA|agCP11536	91	ribosomal protein
1	AD-contig_37	8e–45	CRA|agCP8317	64	ribosomal protein
2	AD-contig_237	6e–70	CRA|agCP14909	84	ribosomal protein
2	AD-contig_226	4e–41	CRA|agCP3608	88	ribosomal protein
1	AD-contig_280	7e–95	CRA|agCP5980	79	ribosomal protein
2	AD-contig_251	2e–43	CRA|agCP6680	68	ribosomal protein
2	AD-contig_257	3e–56	CRA|agCP10609	73	ribosomal protein
2	AD-contig_241	6e–45	CRA|agCP1535	78	ribosomal protein
2	AD-contig_234	6e–84	CRA|agCP10687	94	ribosomal protein
5	AD-contig_126	9e–47	CRA|agCP14068	82	ribosomal protein
1	AD-contig_64	3e–76	CRA|agCP1538	89	ribosomal protein
1	AD-contig_21	1e–40	CRA|agCP14472	87	ribosomal protein
1	AD-contig_132	1e–45	CRA|agCP9994	83	ribosomal protein
1	AD-contig_6	1e–8	CRA|agCP11155	96	ribosomal protein
1	AD-contig_122	1e–102	CRA|agCP9554	82	ribosomal protein
1	AD-contig_33	8e–63	CRA|agCP12827	65	ribosomal protein
1	AD-contig_92	4e–26	CRA|agCP10200	82	ribosomal protein
3	AD-contig_222	9e–78	CRA|agCP12166	97	ribosomal protein
1	AD-contig_113	1e–41	CRA|agCP8681	98	ribosomal protein
1	AD-contig_147	5e–56	CRA|agCP13921	93	ribosomal protein
1	AD-contig_9	1e–76	CRA|agCP12071	89	ribosomal protein
1	AD-contig_98	4e–59	CRA|agCP1749	83	ribosomal protein
1	AD-contig_97	9e–33	CRA|agCP14988	58	ribosomal protein
1	AD-contig_193	1e–104	CRA|agCP7923	93	ribosomal protein
1	AD-contig_4	1e–71	CRA|agCP9782	80	ribosomal protein
1	AD-contig_47	9e–10	CRA|agCP9264	93	ribosomal protein
1	AD-contig_149	2e–41	CRA|agCP3167	81	ribosomal protein

Table 2. (*Continued*)

No. of sequences	Assembled contig	*E* value (best match to NR protein database)	Best match to AGPROT database	Percentage identity	Comments identity (similar to/putative function)
1	AD-contig_96	3e–62	CRA|agCP13543	72	ribosomal protein
1	AD-contig_50	1e–28	CRA|agCP5982	95	ribosomal protein
3	AD-contig_219	1e–32	CRA|agCP4788	47	ribosomal protein
3	AD-contig_223	2e–85	CRA|agCP7766	90	ribosomal protein
1	AD-contig_179	1e–73	CRA|agCP7049	97	ribosomal protein
3	AD-contig_213	2e–54	CRA|agCP11873	92	ribosomal protein
3	AD-contig_225	5e–95	CRA|agCP6972	90	ribosomal protein
2	AD-contig_248	1e–19	CRA|agCP6003	38	ribosomal protein
1	AD-contig_12	8e–17	CRA|agCP4228	62	ribosomal protein
1	AD-contig_93	8e–49	CRA|agCP1091	79	RRM (RNA recognition motif)
1	AD-contig_19	4e–44	CRA|agCP9326	79	Golgi vesicular membrane trafficking protein p18
1	AD-contig_22	2e–73	CRA|agCP11339	93	similar to Reticulon protein 3
1	AD-contig_107	2e–68	CRA|agCP9383	81	probable microsomal signal peptidase 25 kDa subunit
8	AD-contig_54				rRNA, mitochondrial
2	AD-contig_258	1e–5			ribosomal protein
Signalling/transport					
1	AD-contig_56	2e–48	CRA|agCP12773	89	calcyclin, signal transduction
1	AD-contig_185	4e–77	CRA|agCP10575	97	calmodulin
1	AD-contig_79	5e–66	CRA|agCP8333	87	translocase of inner mitochondrial membrane
1	AD-contig_283	4e–68	EBI|7451	76	mitochondrial carrier protein
1	AD-contig_271	1e–9	CRA|agCP12493	65	metallothionein
1	AD-contig_38	5e–28	CRA|agCP6905	91	mitochondrial ATP synthase
1	AD-contig_269	2e–78	CRA|agCP4271	90	myosin regulatory light chain (non-muscle)
1	AD-contig_110	2e–30	EBI|5259	29	Na^+/K^+-exchanging ATPase
1	AD-contig_130	4e–63	CRA|agCP1147	95	GABA(A) receptor-associated protein
1	AD-contig_53	2e–7			adipokinetic hormone
Structural proteins					
1	AD-contig_263	3e–75	CRA|agCP4904	68	integral membrane protein 2A
1	AD-contig_183	1e–102	CRA|agCP5029	94	similar to proteasome
1	AD-contig_176	6e–91	CRA|agCP8637	96	similar to transmembrane 9 superfamily
1	AD-contig_14	0.004	CRA|agCP4724	60	extensin
1	AD-contig_190	2e–62	CRA|agCP4913	32	membrane integral protein
Transcription/translation factors					
1	AD-contig_196	5e–12	CRA|agCP13054	65	elongation factor (super cysteine rich protein)
1	AD-contig_131	3e–58	CRA|agCP13402	90	histone H2A
1	AD-contig_5	3e–22	CRA|agCP6533	79	nuclear regulation
1	AD-contig_282	6e–8	CRA|agCP4557	46	transcription factor
6	AD-contig_112	7e–48	CRA|agCP9704	78	transcription factor, MBF2
1	AD-contig_186	5e–93	CRA|agCP7051	81	translation initiation factor
1	AD-contig_184	1e–111	CRA|agCP2764	94	translation initiation factor 3
1	AD-contig_177	1e–118	CRA|agCP7851	89	phenylalanine-tRNA ligase beta chain

sequenced) are associated with energy metabolism, including several mitochondrial enzymes (cytochromes and ATP synthases) and enzymes from the glucolysis, glucose-6-P, and Krebs cycle pathways (transaldolase, transketolase, aconitase and several dehydrogenases). Eight clusters (thirteen sequences) are associated with possible transcription/translation factors, including elongation factors, transcription-initiation factors, histone H2A and nuclear regulation factor.

Ten clusters are associated with signal/transport transduction pathways including calcyclin, calmodulin, metallothionein, mitochondrial ATP synthase, GABA(A) receptor-associated protein and ATPases involved in ion transport, such as Na^+/K^+-ATPase. Five clusters (six sequences) are associated with products possibly involved in protein folding such as heat shock proteins and chaperonins. Five clusters are associated with cytoskeletal proteins such as integral membrane protein 2 A and extensin.

Description of unknown function (U) category clusters

We sequenced 131 clones, included in ninety-seven clusters (Table 3), and annotated these as unknown sequences. These sequences did not show significant similarity with known proteins and could either represent novel proteins, unique to *An. darlingi* salivary glands, or PCR artefacts (or sequencing errors), and hence they are not described in this work.

Comparison of protein sequence identities between An. darlingi *and* An. gambiae *gene products*

It has been proposed that adult female salivary gland proteins of anopheline mosquitoes and American sand flies are under strong selection owing to the deleterious effect

Table 3. *An. darlingi* salivary glands cDNA clusters with unknown function

No. of sequences	Assembled contig	*E* value (best match to NR protein database)	Best match to AGPROT database	Percentage identity
1	AD-contig_75	2e–13	EBI|7994	85
5	AD-contig_197	0.092	EBI|6164	46
1	AD-contig_118	0.001	EBI|5715	31
1	AD-contig_165	0.088	EBI|5060	84
2	AD-contig_240	3e–13	EBI|4655	23
1	AD-contig_195	0.017	EBI|2778	80
1	AD-contig_83	0.086	EBI|1072	29
3	AD-contig_211	0.059	CRA|agCP9352	70
1	AD-contig_215	1e–9	CRA|agCP8743	51
1	AD-contig_216		CRA|agCP8743	70
1	AD-contig_7	8e–17	CRA|agCP8728	60
5	AD-contig_170	0.050	CRA|agCP8362	33
1	AD-contig_105	8e–7	CRA|agCP8258	40
2	AD-contig_254	0.004	CRA|agCP8099	39
1	AD-contig_80	1e–62	CRA|agCP7842	72
1	AD-contig_27	1e–37	CRA|agCP7057	88
1	AD-contig_117	0.018	CRA|agCP6430	34
3	AD-contig_204	1e–51	CRA|agCP6351	79
1	AD-contig_194		CRA|agCP6071	52
1	AD-contig_20	9e–71	CRA|agCP5466	72
1	AD-contig_49	0.003	CRA|agCP4972	60
1	AD-contig_99	0.094	CRA|agCP4788	100
1	AD-contig_182	6e–12	CRA|agCP4733	50
2	AD-contig_236	1e–57	CRA|agCP4692	60
1	AD-contig_61	0.002	CRA|agCP4376	32
1	AD-contig_188	6e–65	CRA|agCP4171	93
1	AD-contig_91	1e–16	CRA|agCP3793	93
1	AD-contig_264	9e–4	CRA|agCP2750	66
1	AD-contig_145	3e–13	CRA|agCP1820	31
1	AD-contig_175		CRA|agCP1764	33
1	AD-contig_86		CRA|agCP15025	36
2	AD-contig_250	$3e^{-17}$	CRA|agCP13443	24
2	AD-contig_17	0.001	CRA|agCP13284	52
1	AD-contig_205	0.001	CRA|agCP13284	52
1	AD-contig_23	2e–5	CRA|agCP12804	32
1	AD-contig_18	7e–57	CRA|agCP12530	70
2	AD-contig_249	1e–8	CRA|agCP11977	46
2	AD-contig_245	8e–6	CRA|agCP11977	34
1	AD-contig_162		CRA|agCP11977	29
1	AD-contig_155	5e–22	CRA|agCP11977	56
4	AD-contig_210		CRA|agCP11543	42
1	AD-contig_232	0.013	CRA|agCP11536	100
1	AD-contig_142	7e–67	CRA|agCP11064	94
1	AD-contig_25	3e–90	CRA|agCP10880	90
1	AD-contig_260	0.014	CRA|agCP10645	30
6	AD-contig_41	0.024	CRA|agCP10484	23
1	AD-contig_273	0.008	CRA|agCP10434	32
1	AD-contig_187	0.005	no match	
3	AD-contig_212	0.004	no match	
3	AD-contig_218	0.059	no match	
1	AD-contig_138	2e−4	no match	
1	AD-contig_277	0.079	no match	
1	AD-contig_43	0.002	no match	
1	AD-contig_171	0.009	no match	
1	AD-contig_172	0.061	no match	
1	AD-contig_173	0.005	no match	
1	AD-contig_164	0.073	no match	
1	AD-contig_120	0.071	no match	
2	AD-contig_252	0.076	no match	
2	AD-contig_42	0.091	no match	
1	AD-contig_102	0.081	no match	
2	AD-contig_233		no match	
1	AD-contig_101		no match	
1	AD-contig_103		no match	
1	AD-contig_108		no match	
1	AD-contig_109		no match	

Table 3. (*Continued*)

No. of sequences	Assembled contig	*E* value (best match to NR protein database)	Best match to AGPROT database	Percentage identity
1	AD-contig_127		no match	
1	AD-contig_133		no match	
1	AD-contig_139		no match	
1	AD-contig_140		no match	
1	AD-contig_141		no match	
1	AD-contig_143		no match	
1	AD-contig_163		no match	
1	AD-contig_167		no match	
1	AD-contig_174		no match	
1	AD-contig_178		no match	
1	AD-contig_52		no match	
1	AD-contig_62		no match	
1	AD-contig_227		no match	
1	AD-contig_259		no match	
1	AD-contig_270		no match	
1	AD-contig_272		no match	
1	AD-contig_275		no match	
1	AD-contig_70		no match	
1	AD-contig_74		no match	
1	AD-contig_8		no match	
1	AD-contig_84		no match	
1	AD-contig_90		no match	
1	AD-contig_94		no match	
1	AD-contig_95		no match	
1	AD-contig_55		no match	
1	AD-contig_150		no match	
1	AD-contig_152		no match	
1	AD-contig_153		no match	
1	AD-contig_156		no match	
1	AD-contig_157		no match	
1	AD-contig_160		no match	

that vertebrate host immunity has on feeding (Lanzaro *et al*., 1999; Valenzuela *et al*., 2003). However, salivary gland genes involved in blood feeding also may be rapidly evolving to adapt to a different repertoire of hosts (Valenzuela *et al*., 2003). Because the primary host of both *An. darlingi* and *An. gambiae* is human (Deane, 1986; Constantini *et al*., 1998), and these two anophelines belong to different subgenera, we compared their genes belonging to the secreted (S) and housekeeping (H) categories. For this comparison, we used the *An. gambiae* (subgenus *Celia*) protein data set recently submitted to NCBI and *An. darlingi* (subgenus *Nyssorhynchus*) sequences that originated from two or more cDNA sequences and that gave > 100 amino acid residues of match to the *An. gambiae* sequences when these were compared by blastp with the filter removed.

Both the average and the variance (% identity) of the two data sets were significantly different ($P < 0.0001$). The H genes had an average of $80.11 \pm 16.3\%$ identity, whereas the S genes had $47 \pm 14.1\%$ (average $\pm$ SD; averages tested by *t*-test with nonequal variances; variances tested by the *F*-test) (Table 4). We conclude that the salivary gland genes encoding secreted products are rapidly evolving in comparison with the housekeeping genes of these species. Valenzuela *et al*. (2003) found similar results when the salivary glands transcriptomes of *An. stephensi* and *An. gambiae* were compared. These two species belong to the same subgenus (*Celia*) and when compared showed 93% of identity for gene products of the housekeeping group whereas the salivary proteins are only 62% identical. These results support the idea that S genes may be good markers for assessing phylogeny among closely related species, as has been demonstrated with triatomine bugs using the salivary hemeproteins (Soares *et al*., 1998, 2000). Manguin *et al*. (1999) showed weak differentiation among *An. darlingi* populations ranging from Mexico to Argentina. However, previous studies based on behavioural (patterns of biting activity), morphological (body size and polytene chromosome patterns) and molecular (allozymes and ITS2 sequences) differences among geographically distinct populations have indicated the possibility that *An. darlingi* is a complex of closely related species (Lounibos & Conn, 2000). The analysis of salivary gland genes may be a useful tool for further analysis of the *An. darlingi* taxonomic status.

Final remarks

This is the first extensive work of DNA sequence and analysis conducted with a neotropical anopheline mosquito.

Table 4. Identity of amino acids between housekeeping and putative secreted proteins of *An. darlingi* and *An.gambiae* salivary glands

Assembled contig	Comments	Match	% identity
Housekeeping			
AD-contig_246	heat shock protein cognate 4	CRA|agCP12309	95
AD-contig_200	ubiquitin	CRA|agCP13749	59
AD-contig_208	ribosomal	CRA|agCP1641	81
AD-contig_235	ribosomal protein	CRA|agCP14911	87
AD-contig_231	ribosomal protein	CRA|agCP11536	91
AD-contig_237	ribosomal protein	CRA|agCP14909	84
AD-contig_226	ribosomal protein	CRA|agCP3608	88
AD-contig_251	ribosomal protein	CRA|agCP6680	68
AD-contig_257	ribosomal protein	CRA|agCP10609	73
AD-contig_241	ribosomal protein	CRA|agCP1535	78
AD-contig_234	ribosomal protein	CRA|agCP10687	94
AD-contig_126	ribosomal protein	CRA|agCP14068	82
AD-contig_222	ribosomal protein	CRA|agCP12166	97
AD-contig_219	ribosomal protein	CRA|agCP4788	47
AD-contig_223	ribosomal protein	CRA|agCP7766	90
AD-contig_213	ribosomal protein	CRA|agCP11873	92
AD-contig_225	ribosomal protein	CRA|agCP6972	90
AD-contig_248	ribosomal protein	CRA|agCP6003	38
AD-contig_112	transcription factor, MBF2	CRA|agCP9704	78
Average ± SD			80 ± 16.3
Salivary			
AD-contig_214	30 kDa allergen	CRA|agCP8743	58
AD-contig_66	antigen-5	CRA|agCP6145	75
AD-contig_1	D7	CRA|agCP11198	61
AD-contig_230	D7	CRA|agCP11220	36
AD-contig_279	D7	CRA|agCP11196	36
AD-contig_77	D7	CRA|agCP11196	28
AD-contig_159	D7	CRA|agCP10845	50
AD-contig_229	D7	CRA|agCP11220	37
AD-contig_256	D7, short	CRA|agCP11220	28
AD-contig_137	anophelin	CRA|agCP11208	43
AD-contig_253	apyrase	CRA|agCP9757	49
AD-contig_217	apyrase (full clone)	CRA|agCP10591	81
AD-contig_224	apyrase	CRA|agCP10591	54
AD-contig_88	maltase	CRA|agCP12790	58
AD-contig_123	salivary glucosidase	CRA|agCP12065	49
AD-contig_125	salivary glucosidase	CRA|agCP12065	37
AD-contig_242	mucin	CRA|agCP1772	51
AD-contig_244	mucin	CRA|agCP1772	40
AD-contig_220	mucin	CRA|agCP7687	31
AD-contig_266	cecropin	CRA|agCP7505	51
AD-contig_181	cecropin	CRA|agCP7503	50
AD-contig_203	defensin	CRA|agCP6915	78
AD-contig_239	putative infection responsive short peptide	EBI|7267	32
AD-contig_199	gSG1b	CRA|agCP13537	37
AD-contig_28	gSG2	CRA|agCP6138	32
AD-contig_207	gSG7	CRA|agCP11109	44
AD-contig_247	gSG7	CRA|agCP2222	54
AD-contig_202	gSG8	CRA|agCP7185	54
AD-contig_255	SG1 family	CRA|agCP13467	32
AD-contig_201	SG3 family	CRA|agCP6430	42
Average ± SD			47 ± 14.1

Furthermore, this study was conducted with *An. darlingi*, an important malaria vector and the most anthropophilic and endophilic species among the Amazonian anophelines (Tadei *et al.*, 1998). Despite its importance as a malaria vector, only twenty-nine nucleotide sequence and thirteen protein sequence entries were available in the NCBI database prior to this study. The description of the salivary transcriptome of *An. darlingi* (subgenus *Nyssorhynchus*) and its comparison to the information available from previously studies anophelines (*An. gambiae* and *An. stephensi*, subgenus *Celia*) represent an advance in the understanding of the mosquito salivary gland functioning and salivary constitution. The comparative analysis of the transcriptomes of several anopheline mosquitoes, belonging to different subgenera and having distinct primary hosts, may supply better tools for the determination of phylogeny of closely related species, population structure and speciation processes, and ultimately identify genes related to vectorial

capacity and host preference. All of this information is likely to be useful for the improvement of existing and development of novel transmission-reduction malaria control strategies.

Experimental procedures

Mosquitoes and cDNA library construction

Adult female *An. darlingi* were caught in Porto Velho, Rondonia, Brazil, and sent to the Institute of Biomedical Sciences, University of São Paulo, Brazil. PolyA$^+$ RNA was extracted from sixty dissected pairs of salivary glands using the Micro-FastTrack mRNA isolation kit (Invitrogen, Carlsbad, CA, USA), which was then used to make a PCR-based cDNA library using the SMART™ cDNA library construction kit (BD Biosciences-Clontech, Palo Alto, CA, USA) as described by Francischetti *et al*. (2002b).

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) of salivary gland proteins of *An. darlingi* adult females was performed using 1 mm thick, gradient (4–12%), NU-PAGE gels (Invitrogen). Gels were run with MES buffer according to the manufacturer's instructions. To estimate the molecular weight of the salivary gland proteins, SeeBlue™ markers from Invitrogen (myosin, BSA, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin, lysozyme, aprotinin, insulin, chain B) were used.

Salivary glands were treated with NU-PAGE LDS sample buffer (Invitrogen) and fifteen pairs of homogenized salivary glands (approximately 15 μg of protein) were applied per lane. Proteins were stained with Coomassie blue G when required. For amino-terminal sequencing, fifteen pairs of salivary glands were electrophoresed and transferred to a polyvinylidene difluoride (PVDF) membrane using 10 mM CAPS, pH 11.0, and 10% methanol as the transfer buffer on a blotmodule for the XCell II Mini-Cell (Invitrogen). The membrane was stained with Coomassie blue G in the absence of acetic acid. Stained bands were cut from the PVDF membrane and subjected to Edman degradation using a Procise sequencer (Perkin-Elmer Corp., Foster City, CA, USA). To identify the cDNAs encoding the amino acid sequences obtained by Edman degradation, a search program written in Visual Basic (Valenzuela *et al*., 2002c) was used, which checked the amino acid sequences against the three possible protein translations of each cDNA sequence obtained in the *An. darlingi* mass-sequencing project.

cDNA sequence clustering

Randomly selected cDNA clones obtained from the salivary glands cDNA library were sequenced and analysed as in Francischetti *et al*. (2002b) and in Valenzuela *et al*. (2002c), except that clustering of the cDNA sequences was accomplished using the CAP program (Huang, 1992). Accession numbers for sequences originating from the *An. gambiae* proteome (Holt *et al*., 2002) are given as agCP ####, where #### corresponds to the referenced gene product. BLAST searches were done locally from programs obtained at the NCBI FTP site (ftp://ftp.ncbi.nih.gov/blast/executables/) (Altschul *et al*., 1997). The electronic versions of the complete tables (Microsoft Excel format) with hyperlinks to web-based databases and to BLAST results (full versions of the tables presented here) are available at: http://www.ncbi.nlm.nih.gov/projects/Mosquito/A_darlingi_sialome/.

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